Immortalized Avian Cell Lines for Virus Production

The present invention relates to immortalized avian cell lines suitable for production of biologicals or viruses for vaccination. In particular, the cell lines are derived from primary cells which are transformed with at least two viral or cellular genes, one of which causes cell cycle progression whereas the other interferes with innate protective mechanisms of the cell induced by dysregulated replication. The invention moreover relates to the production of said immortalized cell lines and their use for producing biologicals or viruses for vaccination.

Background

Embryonated chicken eggs still are one of the main substrates for the production of human vaccines. They are able to support the replication of a wide range of human and animal viruses. This spectrum includes attenuated viruses, i.e. defective viruses that have impaired potential to replicate in human or mammalian cells and can thus be used as vaccines. Attenuation can be generated or maintained by continuous passage in embryonated eggs. Chicken eggs used for human vaccine production must be certified to be free of a defined set of viral and bacterial contamination (specific pathogen-free or SPF). SPF eggs are available from commercial suppliers. The broad applicability and a long international track record has kept this strategy alive despite clear disadvantages:

SPF flocks of chicken and embryonated eggs are expensive and can constitute up to 40% of the cost of vaccines. Furthermore, it is difficult to continually maintain SPF flocks completely free of pathogens which is evidenced by periodic outbreaks of disease in SPF flocks. A vaccine lot cannot be released until the SPF supplier verifies that the parental chickens for the embryonated eggs used to manufacture the vaccine lot were completely free of any disease. This uncertainty adds a significant cost to the preparation of these vaccines. In pandemic situations with sudden need for a particular vaccine (e.g. influenza) the supply of SPF eggs may be severely limited. In addition, the large-scale

processes for infecting eggs and maintaining virus growth are time consuming and sometimes inconsistent across different vaccine batches.

With the development of cell culture techniques vaccine manufacturers have replaced embryonated eggs with isolated chicken embryonic fibroblasts. While the use of primary cell cultures improves the safety profile, efficiency and reliability of the manufacturing process, it also further increases costs: chicken fibroblasts are prepared from SPF eggs by mincing embryos to establish and amplify viable cells. Typical for primary animal cells the fibroblasts suffer senescence: the doubling time increases with passaging and eventually all cells die. This process occurs after about 20 passages, much earlier than for rodent or some human cell substrates currently used in vaccine manufacture (such as MRC-5 or WI-38). Fibroblast cultures have to be maintained in the presence of 5-10% fetal calf serum, adding additional risk factors to the manufacturing process. They also require a solid surface for propagation and do not grow in suspension, a preferred state for bioreactor applications. Even with the use of multilayer cell factories this substantially limits scale-up procedures. Due to the limited live span a complete set of safety tests has to be applied for each lot of chicken fibroblasts.

Fibroblasts are the only cell type out of the wide variety of different tissues from a chicken embryo that proliferates well. The predominance of fibroblasts compared to other cell types has in some cases decreased theoretical virus yield because in eggs typically the chorioallantoic membrane, an epithelial cell layer, is the main site for virus amplification.

The discussed problems have contributed to severe influenza vaccine shortages in the last two years (2003 and 2004). To overcome these limitations, a permanent cell line growing in a synthetically defined medium, preferably in suspension or at least on carriers, would be highly desired.

Some of the viruses typically grown in chicken fibroblasts have been adapted to certain cell lines. BHK-21 (baby hamster kidney) cells support the growth of various vaccinia, influenza, and rabies vaccine strains (Drexler, I. et al., J. Gen. Virol. 79(Pt2):347-52 (1998); Gumusderelioglu M. et al., Biotechnol. Appl.

Biochem. 33:167-72 (2001); Merten, O.W. et al., Adv. Exp. Med. Biol. 397:141-51 (1996)) and easily grow in large fermenters on carriers under serum-free conditions (Pay, T.W. et al., Dev. Biol. Stand 60:171-4 (1985); Gallegos Gallegos, R. M. et al., Arch. Med. Res. 26:59-63 (1995)). For vaccinia this applies even to the highly attenuated strain Ankara (MVA) which was developed on chicken cells. The BHK-21 cell line is accepted for production of certain vaccines for livestock animals (Lubiniecki, A.S., Bioprocess Technol. 10:495-513 (1990)). However, the BHK-21 line does not meet the safety requirements for human live vaccines. BHK cells have spontaneously formed, are highly tumorigenic and their history is inadequately reported.
According to the FDA, CBER Discussion from May 12 , 2000 on cell substrates the development of "Minimally-Purified Live-Attenuated Viral Vaccines and Virus-Vectored Vaccines" in neoplastic cells derived from naturally occurring tumors from humans and other mammals or from human cells and mammalian cells that have been transformed by unknown mechanisms is discouraged.

As an exception to the rule the VERO cell line (originating from African green monkey) is allowed as a cell substrate for vaccine manufacture based on a proven safety profile and the lack of transformed phenotype for a defined number of passages. The cell line has been used extensively for the manufacture of the polio and smallpox vaccines for clinical use. However, VERO cells require attachment and are amenable only to carrier based processes.

Additionally MDCK cells (a spontaneous cell line from dog kidney epithelium) with a described history have been applied to the manufacture of influenza virus (Tree, J. A. et al., Vaccine 19:3444-50 (2001)).

More recently, triggered by the development of vector based vaccines and gene therapy approaches, new so-called designer cell lines of human origin are intensely discussed and included into the spectrum of potential cell substrates for vaccine production (Vaccines and Related Biological Products advisory committee, session from May 16, 2001). New permanent cell lines were created to provide complementing genes for recombinant viruses that are replication-deficient outside the production system. However, stable introduction of the complementing genes requires prolonged cultivation times, which either exceed

the natural limit of passage numbers available to primary cells or the tolerated limit of passage numbers for VERO cells before full transformation occurs.

Designer cell lines are generated *in vitro* with extensive documentation using characterized genes for transformation. For example, the complementing genes from the E1 region of adenoviruses by themselves exhibit transforming properties and have allowed establishment of human cell lines, for example PER.C6 (Fallaux, F.J. et al., Hum. Gene Ther. 9 :1909-17 (1998)). The application of these cell lines is not limited to the viral vector they are designed for but may be extended to other viruses. For example, influenza virus can be propagated on PER.C6 (Pau, M.G. et al., Vaccine 19:2716-21 (2001)). However, this finding does not apply to all viruses relevant to vaccine development, in particular avian viruses such as Marek's disease, infectious bursal disease, Newcastle disease, turkey herpes, or chicken anemia viruses. While some of these viruses replicate well on mammalian cell lines, virus growth is often poor. For other viruses, replication is poor and limited to particular especially adapted strains.

In addition, with adaptation to a primate-derived cell substrate, receptor binding sites on the virus are likely to change resulting in a modified antigen pattern and thus a general effect on immunogenicity. This genetic adaptation may reverse attenuation for strains which have been developed via passaging in avian cells such as MVA or chicken-adapted measles virus (Escoffier, C., Gerlier, D., J. Virol. 73:5220-4 (1999)), or create new strains replicating more efficiently in human cells compared to their wild type isolates. Such viruses may also obtain a higher pathogenic potential.

For the above reasons vaccine manufacturers are reluctant to switch to mammalian cell lines and a need for immortal avian cell lines has developed.

The investigation of tumor induction in birds by the avian alpharetroviruses provided first molecular insights on cell transformation in general. The retroviral oncogenes are derived from cellular genes with essential regulator domains mutated or deleted. Some of the factors that have been identified in the course of these studies, such as v-myc or v-ras, directly affect components of both

retinoblastoma (RB) and p53 pathways. Other proteins, such as v-src or v-erbB, are constitutively activated (hence, dysregulated) signal transducers that mimic impinging extracellular mitogens. The problem with these factors is that they target only one of several pathways required for efficient transformation. The presence of v-src or v-myc predisposes the cell for transformation and requires additional, spontaneous and unpredictable alterations within the cell for full transformation. The risks for the patient posed by cells transformed with one of the retroviral oncogenes therefore is difficult to estimate.

In other cases a single strong tumor antigen (e.g. v-jun) is able to directly cause tumor formation (Hartl, M. et al., Curr. Cancer Drug Targets 3 :41-55 (2003)). Many avian viral oncogenes maintain their oncogenic potential in mammalian cells.

Cell lines created by these viruses are not suitable for vaccine manufacturing. A retrovirus carrying an oncogene may get activated and transferred together with the vaccine. Even a tumor antigen not enclosed by viral LTRs may pose a high risk when it is able to transform mammalian cells without the help of complementary antigens. This risk is typically estimated by consideration of the transforming potential, the number of vaccinees, and the amount of cellular nucleic acid transferred with the vaccine virus. This amount is limited by the efficiency of the purification process and currently cannot be reduced to below 10 pg/dose. This criterion is especially stringent for vaccine production where a healthy population often is inoculated at a very young age.

The same arguments apply to transforming DNA viruses such as papillomaviruses and polyomaviruses. These viruses are equiped with aggressive oncogenes: SV40 large T antigen is a multifunctional protein which affects both checkpoint control in G1 of the cell cycle and p53 activity. Therefore, large T readily immortalizes and transforms multiple mammalian tissues of rodent and human origin. With the addition of small T antigen (further enhancing large T action and additionally modulating the AKT3 pathway) it was possible to immortalize avian cells (part of patent application US 2001-0016348). However, even with sophisticated modern purification methods SV40 large-T antigen is considered too aggressive for use in cell lines generated for application in human

medicine. In contrast to the above, the genes proposed in this invention affect checkpoint control of the cell cycle and p53 inactivation via separate factors: a required simultaneous transfer event of two distinct factors for transformation dramatically decreases any theoretical risk for the vaccinee.

US patent application 2001-0016348 describes the use of an anti-apoptotic pathway completely unrelated to the present invention. It does not provide a second gene that counters an internal signal for apoptosis due to forced cell cycle progression caused by a first gene. Apoptosis can also be induced by a variety of external simuli, for example lack of growth factors or loss of anchorage. Transmission of this type of pro-apoptotic signal can be inhibited by bcl-2 family genes, the focus of US patent application 2001-0016348.

Whereas 90% of cervix carcinomas carry papillomavirus sequences, C-type adenoviruses (which include types 2 and 5) are considered not to induce tumors *in vivo*, and adenoviral sequences have not been detected in human tumor tissue.

Alternatively, it has been tried to develop cell lines by continuous passaging of chicken embryonic fibroblasts. Whereas rodent cells appear to undergo spontaneous immortalization quite easily (Curatolo et al., In Vitro 20:597-601 (1984)), avian and primate cells are highly resistant to this approach (Harvey, et al., Genes and Development 5:2375-2385 (1991); Pereira-Smith, J. Cell Physiol. 144:546-9 (1990); Smith et al., Science 273:63-67 (1996)). Somatic cells of avian or primate origin lack telomerase and senescence is caused by the shortening of chromosomal ends (telomeres). Nevertheless, a chicken fibroblast line UMNSAH-DF1 has been developed using this approach (US patents 5,672,485 and 6,207,415). Immortalization by this approach is caused by spontaneous mutations in multiple oncogenes or tumor suppressor genes. This is a rare event which is unlikely to be reproduced especially in cells of other tissue origin. Most importantly, such an approach contradicts the Defined Risk approach as a general rule for human live vaccines proposing detailed knowledge about the immortalizing genes to assess the risk of oncogene transfer. Again, according to the FDA (CBER Discussion from May 12, 2000, on cell substrates) the use of neoplastic cells derived from naturally occurring

tumors or cells that have been transformed by unknown mechanisms is discouraged for the development of minimally-purified live-attenuated viral vaccines and virus-vectored vaccines.

The spontaneously developed UMNSAH-DF1 chicken fibroblast line exhibits alterations in E2F and p53 activity (Kim et al., Oncogene 20: 2671-82 (2001)). This is not surprising because enhanced cell cycle activity requires active E2F, and because it is known from mammalian cell studies that high E2F activity induces apoptosis in the presence of active p53. The study characterizes the immortal stage without shedding light on the causative events: mutations in a large number of genes may have caused immortalisation.

A spontaneous transformation process may be enhanced by the use of chemical mutagens (US Patent 5,989,805). The particular cell lines generated using this approach have overcome senescence but maintained a fibroblast like appearance and are non-tumorigenic. Although this represents a significant safety feature, these cells are of low value for large scale fermentation techniques. Furthermore, this chance-based approach also contradicts the Defined Risk guidelines.

Avian cell lines originating from naturally occurring tumors such as a quail fibrosarcoma (WO 97/08307) have also been proposed for biomanufacturing. Again, the Defined Risk guidelines for use in human vaccine production are violated by a method that is based on chance events.

The approaches taken in the studies described above are in sharp contrast to the active introduction of specific groups of immortalising genes according to this invention, which defines the causative agents for immortalisation and allows to assess risk, provides high flexibility with respect to selection of various tissues, and allows to modulate certain features of the resulting cell line.

Despite the fact that chicken eggs and fibroblasts have a considerable track record they are also associated with a very specific risk factor that only recently has come into greater focus: chicken cells release at least two types of retroviral particles, the endogenous avian retrovirus (EAV) and the endogenous avian

leukosis virus (ALV-E). The issue is similar to the presence of endogenous retrovirus particles in mouse cells which are used for the manufacture of recombinant proteins (such as NS0). However, in contrast to mouse cells, chicken cells have been shown to contain reverse transcriptase. Due to more efficient detection techniques RT activity has also been detected in chicken cell-derived measles, mumps and yellow fever vaccines (Hussain, A.I. et al., J. Virol. 77:1105-11 (2003); Shahabuddin, M. et al., J. Clin. Microbiol. 39 :675-84 (2001)). Whether the presence of reverse transcriptase activity results in transmissible retroviruses remains controversial: a more detailed analysis has shown that CEF (from White Leghorn) contain five loci with integrated EAVs, two of which can express infectious ALV-E whereas the other three are defective (Johnson, J.A., Heneine, W., J. Virol. 75:3605-12 (2001)). Tsang, S.X. et al., J. Virol. 73 :5843-51 (1999) also found RT activity and release of viral particles but did not observe any transmission after a careful search for EAV sequences in blood mononuclear cells of children that received mumps vaccine. According to the Weekly Epidemiological Record of the WHO (73) 28 (1998), independent laboratories have investigated the infectivity of the particles for a variety of human and other mammalian cells by extensive co-cultivation and could not detect transmission of RT activity or productive infection. This finding is supported by epidemiological studies that have revealed no association between the use of chicken cell-derived vaccines and incidence of cancers, including those of childhood.

Furthermore, in the mentioned Weekly Epidemiological Record, the WHO stresses the importance of chicken host cells to maintain attenuation of certain vaccine strains. Alternative production processes are not currently available, and this lack of alternatives is an important reason for the acceptance of a known and continous source for a viral contaminant.

However, epidemiological studies superimpose populations and do not investigate chance events or case studies. Epidemiological studies cannot refute theoretical risks, for example: the accepted endogenous RT activity may mask RT activity from unacceptable exogenous contamination, and the endogenous viruses may be mobilized and activated if packaging constructs are introduced into the cells (Ronfort, C. et al., Virology 207:271-5 (1995)).

It was shown, however, that cells from ducks and geese do not contain EAV and ALV related sequence and the Japanese quail is free of reverse transcriptase (Smith, L.M. et al., J. Gen. Virol. 80(pt1):261-8 (1999); Brudno, I.A. et al., Vopr. Virusol. 97-100 (1980)).

Adenoviruses (AdV) are well characterized, naked (non-enveloped) ubiquitous viruses. For the most common serotypes Ad2 and Ad5 the seroprevalence in the human population approaches 90%. Replication incompetent versions of these viruses are used as gene therapy and vaccine vectors in trials with human patients. Genes from the E1 region of human Adenovirus 5 have been used to transform some specific human cells in vitro (293 and PER.C6 cell lines; Fallaux, F.J. et al., Hum. Gene Ther. 9:1909-17 (1998); Graham, F.L. et al., J. Gen. Virol. 36:59-74 (1977)). The general process is inefficient compared to stronger multifunctional oncogenes such as SV40 large T antigen. Based on the observation that 293 show neuron specific markers and PER.C6 are of neuroectodermal origin it was suggested that Ad5 E1-based transformation is limited to neuronal cells (Shaw et al. Faseb J 16(8): 869-71(2002)). Considering the significant species barrier between human and avian cells efficient immortalisation of multiple avian tissues by transfection is even more unexpected.

Mammalian E1 transformed cell lines have been used for the production of live purified adenovirus vectors in clinical trials. With careful monitoring of the amount of contaminating cellular DNA in a vaccine preparation and its size, the transforming genes of Ad5 are not considered a safety hurdle (Vaccines and Related Biological Products advisory committee, session from May 16, 2001).

Adenoviruses replicate in the nucleus of the infected cell. Because quiescent host cells are not permissive for a full viral life cycle adenoviruses have evolved mechanism to force cells into S-phase. To maximize burst size of progeny viruses they have also evolved mechanism to evade apoptosis as a response of the host cell to capsid penetration and viral replication. The genomic region that mediates both cell cycle progression and inhibition of apoptosis is the E1 region.

The E1 region actually consists of two distinct expression cassettes, E1A and

E1B, arranged in tandem and each equipped with its own promoter and polyadenylation site. At least three proteins are translated from the E1A primary transcript by alternative splicing. Among others, E1A proteins have been found to disrupt RB/E2F complexes and to interfere with the p300 and CBP transcriptional co-activators. The escape of E2Fs from the RB repressor induces progression of the cell cycle from G1 to S phase, whereas the E1A/p300 complex induces apoptosis via several pathways (Putzer, B.M. et al., Cell Death Differ. 7:177-88 (2000)), including repression of transcription of MdM2, a negative regulator of the key sensor for apoptosis, p53.

As E1A sensitizes cells to TNF-induced apoptosis it is considered an antitumor agent, and it is used in experimental approaches for tumor treatment (Lee, W.P. et al., Cancer Res. 63:6229-36 (2003)).

Furthermore, acting as a transcription modulator it drives cells towards de-differentiation, a feature advantageous to a potential cell substrate.

It was shown by Guilhot et al. (Guilhot, C. et al., Oncogene 8:619-24 (1993)) that retroviral transduction of the 12S protein of E1A from Ad5 can lead to immortalization of quail cells. This is likely the consequence of interaction between the avian RB and E1A. However, the process fails when the gene is introduced by transfection of naked DNA instead of retrovirus infection (pers. observation). We propose that the extremely efficient and stable transduction via retrovirus infection creates a cell pool large enough to harbor individual cells with spontaneous genomic changes that have blocked apoptosis that normally is induced upon RB inactivation. These required but unknown changes increase the risk for vaccinees and the resulting cell line cannot be considered a designer cell line (the result of defined blocks in specific pathways). Moreover, the transforming gene introduced via retroviruses is flanked by inverted terminal repeats and can, therefore, be mobilized. Such an event may even be more pronounced in cell lines that expresse reverse transcriptase from endogenous retroviruses.

Summary of the Invention

In view of the above, it is still desirable to develop an avian cell line with

convenient growth properties for large scale manufacture, using a defined combination of immortalizing/transforming genes. It is further desirable that none of these genes is able to transform mammalian cells independent of the other genes. Moreover, the action of a single gene should either have no immortalizing/transforming effect or result in apoptosis of cells expressing the respective gene. The risk of joined transfer to a vaccine recipient should further be minimized by positioning the respective genes on separate expression units. Finally, it would be desirable – as the human population is typically exposed to the respective genes - that these genes are not associated with tumor formation in the human population. The cell line to be generated should not release infectious virus particles from endogenous retroviruses or not exhibit reverse transcriptase activity at all.

It was found that transformation of avian cells with two particular viral and/or cellular genes, one of which affecting the retinoblastoma proteins and the other the p53 protein, provided for a cell line well suited for the production of viruses for vaccination.

The invention thus provides:
(1) an avian cell line immortalized with a combination of viral and/or cellular genes (hereinafter shortly referred to as "gene(s)"), at least one first gene affecting the function of the retinoblastoma protein and at least one second gene affecting the p53 protein or a family member thereof, wherein preferably the first gene overcomes G1 checkpoint control and the second gene prevents apoptosis induced by the first gene;
(2) a method for preparing a cell line as defined in (1) above, which comprises transforming/transfecting a starting cell with the first and second gene;
(3) the use of the cell line as defined in (1) above for the production of biologicals or viruses, preferably for the preparation of a vaccine or for gene therapy; and
(4) a method for producing viruses or biologicals using a cell line as defined in (1) above.

Short Description of the Figures

Figure 1: Schematic sections of the expression plasmids used for enhanced

immortalization of primary duck cells (example 2). Polyadenylation signals are omitted for clarity. The alphanumerics at the left are short identifiers for the plasmids. *mPGK* and *hPGK*, *phosphoglycerate kinase promoters of mouse* and human, resp.; ad5, E1-endogenous promoter of Ad5; moCMV, mouse CMV immediate early promoter; tk, herpes simplex virus thymidine kinase promoter; orf 22 and gam1, CELO virus genes; E1A and E1B, adenovirus 5 E1 region genes.

Figure 2: Phase contrast microscopy pictures as example of focus formation in Ad5-E1 transfected duck embryonal liver cells (plasmid 49E). A, initial magnification 4 x to depict a complete focus embedded in senescent primary cells. B, initial magnification 20 x: perimeter of a large round focus of small cells arranged in a compact monolayer visible at the right of the panel, primary cells in advanced senescence towards the left.

Figure 3: Immunofluorescence assay for E1A and E1B 55K proteins (example 3). Upper two rows, mix of plasmid 49E-immortalized and primary duck liver cells; bottom two rows, 293 positive control cells. Left column, phase contrast images; middle column, immunostaining of E1A or E1B 55K proteins as indicated in the images; right column, DAPI stain. The E1B 55K protein characteristically localizes to the cytoplasm and accumulates in aggregates to yield an uneven, spotty distribution. E1A is a nuclear protein. Note the compacted nuclei that stain brightly with DAPI in the transformed duck cells.

Figure 4: Q-PERT assay (quantitative PERTassay) *on cell supernatant for* detection of retroviral activity (example 4). Bold squares, CHO positive control; open squares, water negative control; bold diamonds, chicken embryonic fibroblasts; bold triangles, 293 cell line negative control; grey circles, substrate-only negative control; open triangles, duck liver cells immortalized with plasmid 49E; delta Rn, emission of the reporter dye over starting background fluorescence.

Figure 5: MVA *amplification on some of the described* duck cell lines and CEFp (example 5). Infection was performed with an MOI of 0.1. Titration was performed on VERO cells 48 hours after infection (Example 2). CEFp, primary

chicken embryonic fibroblasts.

Figure 6: serial passaging of MVA on duck retina cells immortalized with plasmid 49E (example 5). Bold squares, burst size; bars, input virus adjusted to an MOI of 0.1. Input virus is given as reference to demonstrate that burst size is independent of experimental fluctuations in cell numbers (which in turn define input virus via MOI).

Sequence Listing – Free Text

SEQ ID NO:	Description – free text
1	Primer VS182
2	Primer VS183
3	Primer VS184
4	Primer VS185
5	Primer VintSA-F
6	Primer VintSA-R
7	Plasmid pEFAd5E1A
8	Plasmid pEFAd5E1BSA
9	Plasmid 49E
10	Plasmid 25F
11	Primer V206
12	Primer V207
13	Primer V208
14	Primer V209
15	RT primer
16	Primer cDNA 1
17	Primer cDNA 2
18	Plasmid 60E
19	Plasmid 36E

Detailed Description of the Invention

"Immortalized", "immortalized cells" and "immortalized cell line" according to

the present invention relates to a cell or cell line which has been transfected/transformed by certain functional DNA sequences conferring the potential for at least 200 passages, preferably unlimited number of passages, i.e. immortality, to the respective starting cells.

A "gene cassette" of the present invention is to be understood as a DNA sequence comprising a gene affecting the function of the retinoblasoma protein, i.e. which directly or indirectly (e.g. after expression) mediates the disruption of complexes between retinoblastoma proteins and E2F transcription factors, and which in addition comprises a viral gene preventing induction of growth arrest and apoptosis by p53 such as the adenovirus E1B 55K protein of all groups, the E6 protein of papillomaviruses, preferably those of the low-risk human papillomaviruses (HPV) (such as HPV1, HPV6 and HPV11, but not HPV16, HPV18), or a cellular gene preventing growth arrest and apoptosis by p53 such as mdm2.

In more detail, the above gene cassette comprises a "first gene" which in a preferred aspect of (1) directly or indirectly (e.g. via cellular inducers) mediates the disruption of complexes between retinoblastoma proteins and E2F transcription factors. This first gene may be a viral gene such as a mastadenovirus E1A, gam1 and orf22 of CELO or E7 of papillomaviruses, preferably of the low-risk human papillomaviruses (such as HPV1, HPV6 and HPV11, but not HPV16, HPV18), or a cellular gene such as a constitutively active CDK4 or an over-expressed D type cycline. The activity of the first gene mediates cell cycle progression usually at the cost of induction of apoptosis or growth arrest with increased passaging.

A "second gene" is present in above gene cassette to counter this effect of the first gene. It prevents apoptosis or growth arrest and preferably acts by inhibiting transcriptional activation by p53 via augmenting the degradation of p53 or converting p53 from a trans-activator to a repressor of transcription. Preferably the "second gene" is capable of preventing transcriptional activation by p53, including repression of the function of p53 and causing a decrease in stability of p53. The "second gene" may be a viral gene such as the adenovirus E1B 55K protein of all groups, orf22 of CELO, the E6 protein of papillomaviruses,

preferably of the low-risk human papillomaviruses (such as HPV1, HPV6 and HPV11, but not HPV16, HPV18), or a cellular gene preventing growth arrest and apoptosis by p53 such as mdm2. Preferably the "second gene" is orf22 of CELO or adenovirus E1B 55k.

This is exactly opposite to the introduction of exogenous active wild type p53 which was associated with the generation of a chicken fibroblast line by an unknown mechanism (US 5,879,924).

"Biologicals" in the context of present invention comprises therapeutic and recombinant proteins, including antibodies, enzymes, hormones, receptors or their ligands and fusions thereof. Prefererred biologicals are recombinant proteins.

One preferred aspect of embodiment (1) is the use of a cell line derived from embryonic or hatched chicken, duck, goose, quail or the like, preferably from chicken or duck. In an especially preferred aspect of (1), additionally this cell line is free of reverse transcriptase activity, derived from immortalization of a primary cell originating from chicken embryos, hatched chicken, duck embryos or hatched ducks, is derived from extraembryonic membrane and/or is cultivated in a chemically defined medium. The medium is preferably free of animal serum.

Another preferred aspect of embodiment (1) is that the cells subjected to immortalization are primary cells including fibroblasts, cells from isolated body segments (somites) or separated individual organs including neuronal, brain, retina, kidney, liver, heart, muscle and extraembryonic tissues and membranes protecting the embryo. Most preferably, the cells are from extraembyonic membranes or retina.

The immortalization leading to the cells of embodiment (1) is preferably effected by non-viral transfection, including, but not limited to, transfection mediated by liposomes, dendrimers or hydroxyapatite ("calcium phosphate") precipitates and electroporation.

Preferably, the first gene in embodiment (1) is a viral gene mediating disruption of complexes between retinoblastoma proteins and E2F transcription factors. This includes, but is not limited to, an adenovirus E1A gene from mastadenoviruses (preferably from mastadenoviruses of group C), an E7 protein of papillomaviruses, preferably from low-risk human papilloma virus (HPV) (such as HPV1, HPV6 and HPV11, but not HPV16, HPV18), an orf 22 gene of avian adenoviruses and/or E43 open reading frames from ovine attadenovirus. Alternatively, the first gene of embodiment (1) is a cellular gene mediating disruption of complexes between retinoblastoma proteins and E2F transcription factors. This includes, but is not limited to, cyclin D1, cyclin D2, cyclin D3 and/or a mutated CDK4 not susceptible to inactivation by p16INK4a.

The second gene of embodiment (1) is preferably a viral gene coding for a protein preventing induction of growth arrest and apoptosis by p53. This includes, but is not limited to, genes coding for the adenovirus E1B55K protein of all groups, GAM-1 of CELO, the E6 protein of papillomaviruses, preferably those of the low-risk HPV (such as HPV1, HPV6 and HPV11, but not HPV16, HPV18). Most preferred are genes coding for the adenovirus E1B55K protein and GAM-1 of CELO. Alternatively, the second gene encodes a cellular protein preventing growth arrest and apoptosis by p53 such as mdm2.

The first gene and second gene of embodiment (1) are preferably either separated spatially by heterologous sequences or located on different nucleic acid segments or plasmids.

In an especially preferred aspect of embodiment (1) the first gene is the E1A and the second gene is the E1B region of an adenovirus from the genus Mastadenovirus, preferably from adenovirus 5. Most preferably said E1A regions have the sequence of bp 1193 to 2309, preferably bp 1239 to 2309, of SEQ ID NO:7 or the sequence complementary to bp 4230 to 3113 of SEQ ID NO:9. Furthermore most preferably said E1B regions have the sequence of bp 1145 to 3007, preferably bp 1197 to 2810, of SEQ ID NO:8 or the sequence complementary to bp 2345 to 550 of SEQ ID NO:9.

In a further especially preferred aspect of embodiment (1) the first gene is orf22

and the second gene is GAM-1 from an adenovirus, preferably from the genus aviadenovirus CELO, which preferably have the sequence represented by the sequence complementary to bp 1252 to 635 of SEQ ID NO:10, and the sequence complementary to bp 3138 to 2290 of SEQ ID NO:10.

In even a further especially preferred aspect of embodiment (1) and (2) the plasmids 36E (SEQ ID NO:19), 37E (figure 1), 49E (SEQ ID NO:9), 25F (SEQ ID NO:10) or 60E (SEQ ID NO:18) are used for immortalization of the cells.

Furthermore, combinations of nucleic acids encoding E1A and/or E1B with GAM-1 and/or Orf22 as defined above are preferred aspects of embodiment (1).

The cell line according to embodiment (1) may additionally carry non-natural functional sequences including, but not limited to, transgenes such as genes complementing deficient viruses (e.g. EBNA1, etc.), promoters (e.g. PGK-, EF1.alpha-, CMV-promoter, E1-promoters of Ad5, tk-promoter etc.), enhancers (e.g. RSV-LTR), selection markers such as neomycin-resistance, puromycin-resistance, etc.. In one preferred aspect the first and second gene are under the control of separate promoters selected independently from PGK-, CMV-, E1- and tk-promoters.

The cell line according to embodiment (1) is in one preferred aspect furthermore suitable for production of biologicals or viruses including vaccine strains (Marek's disease, infectious bursal disease, Newcastle disease, turkey herpes, chicken anemia, influenza, vaccinia (MVA), rubella, rabies viruses, etc.) and recombinant viral vectors (e.g. recombinant MVA or alphaviruses). Most preferred viruses for vaccination are MVA and influenza viruses. The most preferred recombinant viral vector is MVA.

In one aspect of embodiment (1) the cell line is cell line 12A07-A10 (DSM ACC2695) derived from immortalization of duck extraembryonal membrane cells with plasmid 49E (example 2).

Furthermore preferred is the generation of the cell lines according to embodiment (1) under cGMP conditions which renders them suitable for

pharmaceutical application.

The method of embodiment (2) preferably comprises non-viral transfection of the starting cell such as listed above. Most preferred is liposomal transfection, especially transfection by the Effectene reagent.

A preferred use according to embodiment (3) is the use for the preparation of a vaccine or for gene therapy. A viral vaccine strain or gene therapy vector is brought into contact with cells of a cell line according to embodiment (1) so that infection occurs and the virus is amplified by said cells. Continued passaging of virus (repeated cycles of infection and harvest of virus on said cells) will lead to attenuation or adaptation of virus to this particular host cell line. Thus, a viral vector or vaccine strain with lesser virulence for the intended vaccinee (which is not duck, preferably not avian) is generated. Attenuated viruses allow the immune system of the vaccinee to launch a response that is more protective than vaccination with fully inactivated particles, and that is less severe than infection with a wildtype (natural) pathogen. The preferred viruses for this embodiment are measles and rabies viruses.

The method for producing viruses according to embodiment (4) preferably comprises the contacting of said viruses with a cell line according to embodiment (1) and/or the cultivation of said viruses on said cell line. Especially, this method can be used for producing a pox virus, preferably strain MVA, in a duck cell line, preferably a cell line originating from duck somites or duck neuronal tissue, even more preferred from duck retina. Especially duck retina and somite-derived cells obtained by transfection of Ad5-E1 region under cGMP conditions stably support amplification of MVA with an efficiency comparable to or better than primary chicken embryonic fibroblasts (Example 5).

The method for producing biologicals, especially recombinant proteins, according to embodiment (4) comprises the introduction of a gene coding for a recombinant protein, operably linked to a promoter into a cell line according to embodiment (1), cultivating said modified cell line and harvesting the recombinant protein.

The method of embodiment (4) is used preferably for the production of viruses and biologicals usable for vaccination or gene therapy.

Historically, chicken eggs and the respective cells (chicken fibroblasts) are the dominating substrate for the manufacturing of vaccines. For pharmaceutical purposes chicken are available from pathogen-controlled environments with an extensive monitoring system. A large body of literature suggests chicken eggs as the primary target for cell line development. Therefore, chicken cells are one preferred source for starting cells of the invention. However, chicken-derived cells and cell lines will be most likely RT positive. Literature data suggest a low risk for release of infectious virus. However, the absence of transmissible virus will have to be monitored for any cell line to be used in manufacturing. Indeed, most of the avian cell lines established so far are originating from chicken (US 5,830,723, US 5,879,924). Although it was possible to breed a chicken lineage (line 0) free of avian leucosis virus, endogenous avian retroviruses (EAV-HP) (Boyce-Jacino et al., J. Virol 66(8):4919-29 (1992)) are present in chicken cells including line 0. EAVs provide an active reverse transcriptase, but expression levels vary substantially. Therefore, even primary chicken cells and cell lines such as DF1 that tested RT negative in less sensitive assays (Crittenden et al., Virology 57(1):128-38 (1974)) presumably will test positive in modern real time PCR approaches and may harbor retroviruses that are activated under certain growth conditions.

Alternatively preferred avian species of this invention for cell line development are those which do not contain endogenous retroviruses or express reverse transcriptase (RT). This includes ducks, which are suitable for two additional reasons: Duck eggs are also available from pathogen free monitored stocks and ducks are, in contrast to geese, less likely to develop spontaneous tumors. While it is known that many of the relevant vaccine strains replicate well in duck (embryonal) cells as they do in chicken (embryonal) cells (e.g. Marek´s disease virus (Witter, R.L., Avian Dis. 46:925-37 (2002)) or rubella (Rocchi, G., Salvadori, A., Nuovi Ann. Ig Microbiol. 21:336-40 (1970))), this remains to be shown for virus strains of primary interest. For other vaccines such data is not available.

To our knowledge it is a novel and unexpected finding of this invention that the highly attenuated pox virus strain MVA (modified vaccinia Ankara) replicates in duck cell lines at similar or higher efficiencies than in commonly used primary chicken embryonic fibroblasts. One intention of the inventors was to provide a safe and robust alternative to primary cells for amplification of viruses that require an avian host, or vaccine strains where a non-mammalian host is preferred. An important virus for which convenient host cells are not available is MVA (modified vaccinia virus Ankara). MVA is a highly attenuated pox virus and an extremely promising tool for therapeutic and protective vaccine applications. MVA will serve as a model virus for characterization of duck cells but should not be taken as an exclusive example: the described experiments can also be performed with a range of other viruses, whether pathogens or therapeutic vectors, such as measles, rubella, rabies, or influenza viruses.

Fibroblasts have been selected as the preferred cell type mainly for historic and practical reasons. Fibroblasts are the fastest growing primary cells from mammalian as well as avian species. When a cell suspension from whole chicken embryos is brought into culture, this is not the only but the predominant cell type. However, fibroblasts grow strongly adherent and loose this feature only after complete (tumorigenic) transformation. This process requires the presence of strong transforming genes such as v-ras interfering with signal transduction pathways. Early senescence of fibroblast cultures is in part caused by the total absence of telomerase activity in birds and man (Forsyth, N. R. et al., Differentiation 69 (4-5):188-97 (2002)).

Human primary fibroblasts are refractory to transformation with the E1 genes of adenovirus type 5 which do not directly interfere with these pathways (personal observation). Efficient immortalization and growth in suspension culture has a higher chance to succeed for epithelial and neuronal cells. Moreover, epithelia instead of fibroblasts seem to be the primary site for virus replication inside the bird egg. Interestingly, in contrast to the human situation, bird kidney does express telomerase throughout life which makes bird kidney cells a good target for immortalization. Taken together, bird epithelial cells including kidney epithelium and neuronal cells are considered the most promising targets to develop a cell line of the required features.

It is therefore only for the ease with which fibroblasts are obtained that avian cell line development has almost exclusively focused on these cells (Cowen, B. S., Braune, M.O., Avian Dis 32(2):282-97 (1988); US 5,830,723). In some cases whole embryos have been used (US 2001-0016348).

Viruses do not only exhibit species but also organ and tissue specificity based on receptor distribution and cellular factors supporting replication. Therefore, in contrast to the typical approach, a preferred way to perform present invention is the separation of organs prior to cultivation to obtain a most preferred host cell.

For influenza virus, whose vaccine-adequate production is a major application for the cell lines of present invention, the typical site of replication is not the embryo itself but extraembyonic membranes. Therefore, a specific aim was to also develop cell lines from extraembryonic material, including protective membranes of the embryo. Some tissue specific primary cultures including those of the extraembryonic membranes have very short survival times compared to fibroblasts. This further highlights the need for designed immortalization to obtain optimized host cells. Successful immortalisation of multiple tissues in a limited time window requires the specific combination of genes used within present invention.

It was not known which of the avian tissues has the highest replicative potential for pox viruses such as MVA or Canarypox. The typical manufacturing process for MVA involves a mixture of cells from an embryo excluding the head which is removed prior to disintegration. It is therefore completely unexpected that a cell line of neuronal origin, developed from the retina, has such a high capacitiy for MVA replication whereas other tissues have not.

The same tissue specificity applies to protein production. The transcriptional capacity is dependent on the available set of transcription factors and even strong ubiquitous viral and cellular promoters exhibit variable strength in different tissues. Moreover, yields of secreted protein strongly depend on the capability of a particular cell type to fold and process (e.g. glycosylate) the protein properly.

The mechanisms leading to immortalization and transformation of primary cells have been well described (Hahn, W.C. et al., Nature 400:464-8 (1999)). Required elements interfere with (1) control of cell cycle progression, (2) programmed cell death induced by the deregulated cell cycle, (3) growth factor signal transduction and for human and avian cells (4) shortening of the telomeres, the linear termini of the chromosomes. A large number of factors are known that can drive primary cells to an immortalized and transformed phenotype but immortalization comes at the cost of inhibiting cellular checkpoints that are responsible to minimize tumor formation in the host. It is therefore desired to select transforming factors that can effect experimental generation of a cell line but pose a minimal risk of tumor induction in the recipients of biologicals derived from the designer cells. This requirement needs to be balanced with the strength of the transforming factors: they should be strong enough to cause transformation without the need for accumulation of additional spontaneous mutations; that is, the molecular pathway leading to the resulting cell line should be known completely (categories I and II according to the FDA CBER Office of Vaccine's presentations at the May 2000 Advisory Committee). It is furthermore desired to select a synergistic combination of factors that individually cannot transform primary cells so that a concurrent transfer of genetic material is required which further minimizes the risk of inadvertent transformation in vaccinees or patients. Finally, it is desired that the transforming factor elicits an immune response in the recipient of biologicals so that immune tumor surveillance is activated in the unlikely event of tumor formation due to product application. The last criterion can be realized if non-cellular but foreign, for example viral, transforming proteins are utilized.

It was now found that the E1 region from human adenovirus 5 (Ad5) is ideally suited to transform avian cells so that the resulting designer cell complies with all of the above criteria.

The E1B region encodes two open reading frames on a bicistronic mRNA, the 21K and 55K proteins. The 55K protein binds to p53 and thus turns the pro-apoptotic transcriptional activator into a repressor. The 21K protein comple-ments this anti-apoptotic activity by binding to Bax, thus maintaining integrity of

the mitochondrial membrane and preventing the release of cytochrome C. This protein is essential to drive adherent cells towards substrate independent growth and hence is essential to a fermentation process in suspension.

It has not been shown before whether human adenovirus E1B 55K can affect the avian homologues of p53. Furthermore, the avian adenoviruses are not equipped with genes resembling E1B so that inference also was not possible. Contrary to all expectations, the inventors have found that E1B can provide the essential functions to allow immortalization by E1A.

A novel and crucial factor for the here described achievement was removal of E1B from its weak natural context and placement under control of a strong, recombinant promoter. This novel modification and combination allowed efficient immortalization of multiple tissues from duck and chicken by transfection instead of retroviral transduction.

Although the underlying mechanism for transformation by E1 is complex one hallmark is a most desirable feature: E1A is a strong inducer of cell proliferation and apoptosis whereas E1B proteins efficiently interfere with apoptosis but cannot release restriction on cell cycle control.

Hence, not a single factor but the continuous presence of E1A and E1B proteins are required to sustain the experimentally induced transformed phenotype.

Since the description of v-src in the 1970s (Brugge, J.S., Erikson, R.L., Nature 269:346-8 (1977)) a panoply of transforming factors have been discovered and characterized. Indeed, it was the study of induction of tumors in birds by alpharetroviruses that provided first molecular insights (Martin, G.S., Nature 227:1021-3 (1970)). The retroviral oncogenes are derived from cellular genes with essential regulator domains mutated or deleted. Some of the factors that have been identified in the course of these studies, such as v-myc or v-ras, directly affect components of the RB and p53 pathways. Other proteins, such as v-src or v-erbB, are constitutively activated (hence, dysregulated) signal transducers that mimic impinging extracellular mitogens. The problem with these factors is that they target only one of several pathways required for efficient

transformation. The presence of v-src or v-myc predisposes the cell for transformation and requires additional, spontaneous and unpredictable alterations within the cell for full transformation. The risks for the patient posed by cells transformed with one of the retroviral oncogenes therefore is difficult to estimate.

Other DNA viruses such as papillomaviruses and polyomaviruses are also known to transform cells in vitro. However, the selected transgenes should not be too aggressive to minimize the risk of tumor induction in the recipients of biologicals via inadvertently transferred cellular DNA. This criterion is especially stringent for vaccine production where a healthy population often is inoculated at a very young age. Even with sophisticated modern purification methods polyomavirus Large-T antigen is considered too aggressive for use in cell lines generated for application in human medicine. Whereas 90% of cervix carcinomas carry papillomavirus sequences (Munoz, N. et al., N. Engl., J. Med. 34816):518-27 (2003)) C-type adenoviruses (which include type 2 and type 5) are not considered to induce tumors *in vivo* and adenoviral have not been detected in human tumor tissue.

Based on the complementary features of the transforming genes shown above, it was found that a combination of genes each interfering with single pathways in the cell cycle and apoptosis is necessary to obtain a genetically stable cell line growing in suspension.

It was shown that the complete E1 region of adenovirus 5 can fulfill these requirements. Whereas it was shown, that the 12S protein of E1A from Ad5 can interact with avian RB (Guilhot, C. et al., Oncogene 8:619-24 (1993)) the functional activity of 55K and 21K proteins in avian cells is demonstrated for the first time in present invention. It is not surprising that some clones of quail cells expressing the 12S protein of E1A exhibit transformed features (Guilhot, C. et al., Oncogene 8:619-24 (1993)). The extremely efficient and stable transduction via retrovirus infection creates a large enough cell pool to allow individual cells to overcome the cell cycle block or induction of apoptosis by spontaneous genomic changes. These required but unknown changes increase the medicinal risk and the resulting cell line can not be considered a designer cell line, which should be

based on known genes. Moreover, transfection techniques are not sufficient to create the large clone pool required for natural selection. Instead retrovirus transduction was required. The transforming gene introduced via this approach will be flanked by ITRs and can, therefore, be mobilized, even more in a cell line expressing reverse transcriptase.

Recently, an avian adenovirus, termed fowl adenovirus type 1 strain CELO (for chick embryo lethal orphan), has been described in greater detail (Chiocca, S. et al., J. Virol. 70:2939-49 (1996)). Large, central genomic stretches of CELO are homologous to Ad5 but differ in important aspects - among others, CELO is not equipped with an E1-homologous region. Furthermore, CELO cannot complement Ad5 mutagenized in E1A and, conversely, Ad5 E1 proteins cannot trans-activate transcription of delayed-early CELO genes (Li, P. et al., J. Gen. Virol. 65(Pt 10):1817-25 (1984)). And yet, CELO is capable to transform hamster cells in vitro (May, J.T. et al., Virology 68:483-9 (1975)). Genes interfering with cell cycle and apoptosis, orf22 and GAM-1, have been identified in the CELO virus (Lehrmann, H., Cotton, M., J. Virol. 73:6517-25 (1999)). Orf22 encodes a protein that interact with RB, and GAM-1 interferes with apoptosis in a fashion similar to the prototypical 21K protein (Chiocca, S. et al., J. Virol. 71:3168-77 (1997)).

It was now found that the genes orf22 and GAM-1 from CELO virus are suitable substitutes for E1A and E1B. The spectrum of available transgenes for transformation of avian cells is therewith expanded. These proteins have not been used previously to transform avian cells.

Furthermore, one of the viral genes may be replaced by a cellular gene. Candidates for such replacement are E2F family members or D group cyclins for the E1A region of adenovirus and mdm2 for the E1B region.

The following cell lines were deposited at the DMSZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany:

1. PBG04 as DSM ACC2577, deposited on September 18, 2002;
2. 12A07-A10 as DSM ACC2695, deposited on October 20, 2004.

The invention will be explained in more detail by reference to the following Examples, which are, however, not to be construed as to limit the invention.

Examples

Example 1: Immortalization of primary duck cells with Adenovirus 5 E1A,B

The adenovirus sequences for E1A and E1B were amplified from the culture of passage 8 of the first generation (E1 deleted) adenovirus Admuc grown in HEK 293 which was heavily contaminated with wild type virus using provestart polymerase (Qiagen).

The following primers were used:

VS182 ACTCGAGCTGACGTGTAGTGTATT (SEQ ID NO:1)

VS183 CACACGCAATCACAGGTT (SEQ ID NO:2)

to amplify the E1 A region and

VS184 ACTCGAGTCATGGAGGCTTGGGAGT (SEQ ID NO:3)

VS185 ACACATTTCAGTACCTCA (SEQ ID NO:4)

to amplify the E1 B region. Both fragments were first cloned into pPCR4blunttopo (Invitrogene).

The E1B construct misses the splice acceptor from the E1B message. It was therefore replaced by a synthetic one amplified using primers from the leader intron of a human immunoglobulin heavy chain. As template, the genomic DNA from PBG04 (DMSZ ACC2577), a murine-human heterohybridoma was used.

Primers:

VintSA-F AAGGTACCCTCCCTAGTCCCAGTGA (SEQ ID NO:5)

VintSA-R CAATGTACAGAGTGGGCTCCTGTGG (SEQ ID NO:6)

This splice acceptor was directly cloned into pEFmyc, containing aEF1 alpha promoter and the myc leader peptide to create fusion proteins. The E1A region was removed from ptopoE1A using EcoR I and Xho I sites and cloned into

pEFmyc directly, removing the myc leader sequence and fusing the E1A to the bovine growth hormone poly A. The E1B region was again removed with EcoR I and Xho I restriction enzymes and cloned into pEFmycSA containing the heterologous splice acceptor site. The resulting plasmids were named pEFAd5E1A (SEQ ID NO:7) and pEFAd5E1BSA (SEQ ID NO:8).

Embryonated duck eggs were incubated at 37°C, 60% air humidity, for 12 days (older embryos yielded more cells but also contained a higher number of contaminating, differentiated fibroblasts). The shell was sterilized with 70% isopropanol, opened at the large end, and the embryo was removed aseptically to a sterile petri dish. The fetal brain and kidneys were removed, transferred to separate petri dishes filled with trypsin/EDTA and minced. After a brief incubation a suspension thereof was mixed with an excess of F12 medium (Gibco/Invitrogen) supplemented with 10% fetal calf serum (Biochrom) and 2% Ultroser G (Ciphergen). This suspension was transferred into a petri dish and cultivation was performed at 37°C (which is lower than the 41.6°C physiological temperature of chicken) and 5% CO_2. The culture medium with non-adherent debris was replaced the following day and cultivation continued until at least 5 x 10^5 cells per 3.5 cm dishes were available for transfection of plasmids pEFAd5E1A and pEFAd5E1BSA.

Initial experiments comparing liposomal (Effectene; Qiagen) and dendromeric (Polyfect; Qiagen) transfection reagents suggested best efficiencies with Effectene. Transfection there was performed using the Effectene reagent; briefly: 2 µg of plasmid DNA was diluted in 200 µl EC Buffer containing 16 µl Enhancer. After an incubation time of 5 min, 16 µl Effectene was added. After an incubation time of 10 min, supernatant was removed from the culture in 3.5 cm dishes and replaced with 1 ml fresh medium containing the transfection mix. After an incubation time of 2 hours at 37°C and 5% CO_2, additional 2.5 ml fresh medium was added to the culture.

The transfected cells were allowed to reach confluency, trypsinated, resuspended in FCS/Ultroser G-supplemented F12 medium, and re-seeded into two 6 well plates (corresponding to a 12-fold expansion). After 5 and 10 days, the medium was replaced with F12 supplemented only with 5% FCS. The plates were scanned

for the appearance of foci of cells with changed morphology (decrease in overall cell size, increased size of nucleus, increased visibility of plasma membranes under phase contrast) and increased confluency.

Approximately 14 days post transfection, once the foci reached a diameter of 1-3 mm the medium was aspirated and the culture washed twice with trypsin/EDTA (Gibco). Trypsin-soaked cloning disks (Sigma) were placed on top of the aspirated foci for 3 min, then transferred into wells of a 24-well plate filled with 500 µl of F12 medium supplemented with 5% FCS.

The cloned, transformed cells were allowed to proliferate until confluency, trypsinized, resuspended in F12 medium supplemented with 5% FCS and transferred into 6-well plates. Once the culture reached confluency in the 6-well plate the cells were transferred to T25 flasks for continuous passaging.

For cryopreservation at defined intervals cells were trypsinized, resuspended in F12 medium containing 5% FCS, collected by centrifugation at 100 g for 10 min, resuspended in F12 medium containing 50% FCS and 10% DMSO (Sigma) to a concentration of approximately 3×10^6 cells per ml, and placed in cryovials in an isopropanol-based cooling device at -75°C. The cooling device ensures a constant cooling rate of 1°C per min. After 24 hours the cells were transferred to liquid nitrogen for permanent storage.

Example 2: Improved preparation of immortalized avian cell lines

a) Preparation of primary cells

The flock of origin for the duck eggs was certified to be free of *Salmonella enteritidis* and *S. typhimurium*; *Mycoplasma gallisepticum* and *M. synoviae*; cases of leucosis, reticulo-endotheliosis, psittacosis, avian influenza, duck hepatitis, and Derzsy´s disease. The animals intentionally were not vaccinated against parvovirus and no cases of parvovirosis were detected. Animals in the flock of origin have been vaccinated against *S. enteritidis* and *S. typhimurium*; *Pasteurella multicodica*; the metapneumovirus Turkey rhinotracheitis; and the paramyxovirus causing Newcastle disease.

The eggs were allowed to equilibrate without agitation at room temperature and

after two days were incubated at 38°C in a damp chamber, rotated frequently by alternating +45° and -45°.

Duck embryos were sacrificed for isolation of primary cells after one or three weeks of incubation. Eggs were transferred to a cGMP unit (a closed laboratory performing as outlined by the Current Good Manufacturing Practices) and the shell was sterilized by wiping with 70% isopropanol under a laminar flow hood. All subsequent steps were performed in the GMP unit under sterile conditions with defined solutions or media.

Eggs were opened carefully, embryos transfered to a large petri dish and killed immediately by decapitation. Samples from the following organs were removed: brain, retina, liver, esophagus, heart, and extra-embryonic membranes.

In addition, cells from somites were prepared from an 8-day-old embryo.

All samples were rinsed with PBS (phosphate buffered saline; Gibco/Invitrogen, USA), treated with trypsin (Gibco/Invitrogen, USA) for 1 to 10 min, and triturated in DMEM:F12 culture medium (Gibco/Invitrogen, USA) supplemented with 10% FCS (Biochrom AG, Germany) by repeated passaging through an 18G syringe. The homogenized samples were cultivated at 37°C and 5% CO_2. Debris was removed from adherent cells by change of medium the following day.

b) Plasmid Constructions

Expression plasmids for E1A, E1B, Orf22, and Gam1 were constructed by extraction of the relevant target regions from the genomic DNA of adenovirus serotype 5 or chicken embryo lethal orphan (CELO) wildtype virus, respectively, by PCR and insertion into vectors equipped with human or mouse phosphoglycerate kinase (hPGK or mPGK), mouse CMV (moCMV) or tk promoters (figure 1).

The adenovirus sequences for E1A and E1B were amplified from wild type virus using ProofStart polymerase (Qiagen, Germany). The following primers were used:

VS182 ACTCGAGCTGACGTGTAGTGTATT (SEQ ID NO:1)
VS183 CACACGCAATCACAGGTT (SEQ ID NO:2)

to amplify the E1 A region and

VS184 ACTCGAGTCATGGAGGCTTGGGAGT (SEQ ID NO:3)
VS185 ACACATTTCAGTACCTCA (SEQ ID NO:4)

to amplify the E1 B region. Both fragments were first cloned into pPCR4-Blunt-TOPO (Invitrogene, USA).

The E1B construct misses the splice acceptor from the E1B message. It was therefore replaced by a synthetic one amplified using primers from the leader intron of a human immunoglobulin heavy chain. As template, the genomic DNA from PBG04 (DMSZ ACC2577), a murine-human hetero-hybridoma was used.

Primers used for amplification:

VintSA-F AAGGTACCCTCCCTAGTCCCAGTGA (SEQ ID NO:5)
VintSA-R CAATGTACAGAGTGGGCTCCTGTGG (SEQ ID NO:6)

The genes GAM-1 and ORF-22 were amplified from wild type CELO virus with primers

V206 AAC CTC GAG ACC CCC CTG TAC ATT CTA (SEQ ID NO:11)
and V207 GCC GTT AAC TTC AGG GAT TGG TTA CAG (SEQ ID NO:12), and

V208 CAC CTC GAG TCC GGA TTA AGA TGA ACG (SEQ ID NO:13)
and V209 CCA GTT AAC AGG TGA ACC ATT TAT ACA G (SEQ ID NO:14), respectively.

Representative examples for the resulting plasmids are given with plasmid 49E (adenoviral factors under control of human PGK and mouse CMV promoters; SEQ ID NO:9), plasmid 25F (CELO factors under control of mouse and human PGK promoters; SEQ ID NO:10), plasmid 60E (adenoviral factors under control

of human PGK and tk promoters; SEQ ID NO:18) and plasmid 36E (CELO factor under control of mouse PGK promoter; SEQ ID NO:19) (see also Figure 1).

Integrity of the expression plasmids was confirmed by sequencing. The plasmids are not equipped to express resistance factors against antibiotics (such as ampicillin) in eukaryotic cells.

c) Transfection

Primary cultures were transfected with expression plasmids for E1 or Orf22/Gam1 shortly after isolation or after single subcultivation. Depending on the experiment, plasmids were transfected as supercoils or after linearization with the Sca I (New Englands Biolabs, USA) restriction enzyme. Initial experiments comparing liposomal (Effectene; Qiagen, Germany) and dendromeric (Polyfect; Qiagen, Germany) transfection reagents suggested best efficiencies with Effectene. Transfection was performed as follows: 2 µg total DNA was diluted into 200 µl provided EC buffer and mixed with 16 µl provided enhancer. After an incubation for 2-5 min at room temperature 20 µl Effectene reagent was added. After 5-10 min at room temperature this mixture was applied to the cells in a 8 cm^2 dish under 1 ml culture medium. After 2-5 hours an additional 1.5 ml culture medium was added. On the following day, the medium was replaced with 2 ml fresh culture medium, and thereafter once per week. Successful transfection was confirmed in parallel experiments with a reporter gene.

The cells were continously passaged in DMEM:F12 medium containing 10% FCS.

Twenty days after transfection changes of morphology in defined subpopulations (foci; figure 2) of some cultures were observed; in other cultures foci did not appear or were not able to compete with robust proliferation of the primary cells; again other cultures suffered massive cell death and senescence shortly after transfection.

A large number of independent foci were expanded from plasmid 49E-transfected cultures with cells derived from liver, retina and extra-embryonic membrane. At passage 10, e.g., cell line 12A07-A10 derived from duck

extraembryonal membrane cells transformed with plasmid 49E was isolated and deposited at the DSMZ.

Foci were also obtained from plasmid 60E-transfected cultures with cells from retina and somites.

In plasmid 49E, PGK and mouse CMV promoters drive expression of E1A and E1B, respectively. Plasmid 60E (SEQ ID NO:18) also encodes the full Ad5-E1 region but expression of the protective E1B region is driven by tk, i.e. a promoter that is not as strong as the mouse CMV promoter (but stronger than the native E1B promoter). Consistent with the protective effect conferred by E1B far fewer foci in fewer cell samples were obtained with this construct when compared to the results with plasmid 49E.

Formation of foci with both primary cell appearance and transformed phenotype was also observed in cultures of liver transfected with CELO plasmids 36E (SEQ ID NO:19) and 25F (SEQ ID NO:10).

Cultures with foci were expanded by treatment with trypsin for 2-3 min and resuspension in DMEM:F12 medium for transfer to fresh culture vessels.

For cryopreservation at regular intervals cells were removed with trypsin, resuspended in DMEM:F12 medium containing 10% FCS, collected by centrifugation at 200 x g for 10 min, resuspended in DMEM:F12 medium containing 50% FCS and 10% DMSO (Sigma, USA) to a concentration of approximately 3 x 10^6 cells per ml, and cooled with a rate of 1°C per min to -80°C. After 24 hours, the cells were transferred to liquid nitrogen for permanent storage.

Example 3: Immunofluorescence assay for stable transfection

Cultures of potentially immortalized cells were seeded on glass slides and allowed to proliferate for several days before fixation with ice-cold methanol for 10 min. The fixed cells were incubated with antibodies against E1A and E1B 55K proteins, secondary antibodies, and fluorescent dye specific against the latter according to standard immunofluorescene methods (Becton Dickinson, UK,

#554155 antibody against E1A, diluted 1:30; Oncogene, USA, #DP08-100UG antibody against E1B 55K, diluted 1:30; secondary antibody directed against mouse or rat, respectively, and conjugated to biotin, both from Jackson Immuno Research, USA, diluted 1:80; visualization with Jackson Immuno Research, USA, #016-070-084 streptavidin-Texas Red conjugate, diluted 1:100). Primary cells still abundant in early, not yet fully established immortalized cell lines and readily distinguishable by morphology provided a convenient internal negative control for antibody specificity. 293 cells (human embryonic kidney cells) that stably express the Ad5 E1-region served as positive control. DAPI (4',6-diamidino-2-phenylindol; Sigma, USA) to 1 µg/ml was added in the final incubation step to stain the nuclei of the cells for orientation purposes.

A strong signal for E1A and 55K was observed only in cells that underwent characteristic changes in morphology confirming successful immortalization by the transfected plasmids (figure 3). Furthermore, spontaneous transformation, a formal possibility, was not observed as all cells with altered phenotype were E1-positive. None of the cells with primary phenotype expressed E1-proteins. Although possible in transfections of supercoils where the linearization of plasmid in the process of integration occurs at random positions none of the examined foci exhibited E1A expression in absence of E1B expression, further emphasizing the requirement for dual pathway disruption for immortalization.

Example 4: Assay for endogenous and exogenous retroviruses

A common problem encountered when vaccines are produced in primary chicken fibroblasts is contamination with exogenous or endogenous retroviruses. The diversity of the retrovirus family is too complex to predict whether a given species is a carrier for retroviruses. Reports from the literature therefore usually are limited to a subset of the retrovirus family, for example EAV-HP/ALV subgroup J (Smith, L.M. et al., J. Gen. Virol. 80(pt1):261-8 (1999)), and then only to a subset of avian species.

A reliable confirmation of contamination with retroviruses therefore should focus on a common motif present in these viruses. Sequence diversity precludes nucleic acid-based detection methods. However, common to all retroviruses is the presence of the reverse transcriptase enzyme. The supernatant of expanded

foci from duck liver cells immortalized with plasmid 49E was therefore assayed by quantitative probe-based product enhanced PCR for reverse transcriptase (Q-PERT) and compared to several controls, *inter alia* CHO as positive control and 293 cells as negative control (see below and figure 4), to detect both endogenous retroviral activity or contamination with free retroviruses. The assay is a modification from the literature (Lovatt, A. et al., J. Virol. Methods 82(2): 185-200 (1999)). Briefly: retroviruses were enriched from culture supernatant by ultracentrifugation with 100000 x g through a barrier of 20% sucrose in PBS to remove cellular debris. Virions (if present) were resuspended into lysis buffer (50 mM Tris pH 7.8, 80 mM KCl, 2.5 mM DTT, 0.75 mM EDTA, 0.5% Triton X-100) and mixed with substrate buffer (10 mM each of dATP, dCTP, dGTP, and dTTP; 15 µM specific primer [GCC TTT GAG AGT TAC TCT TTG; SEQ ID NO:15]; and 0.5 mg/ml fragmented herring sperm DNA [Promega Corp, #D1811]) containing a model RNA (5 µg/ml Brome Mosaic Virus RNA [Promega Corp, USA, #D1541]) that is reverse transcribed if RT activity is present in the sample. cDNA from the model RNA is amplified by PCR with primers (AAA CAC TGT ACG GCA CCC GCA TT; SEQ ID NO:16) and (GCC TTT GAG AGT TAC TCT TTG; SEQ ID NO:17) and detected via SYBR green fluorescence in an AB 7000 Sequence Detection System using the QPCR SYBR Green ROX Mix #AB-1163 from Abgene, UK, according to the instructions of the manufacturer.

Figure 4 demonstrates strong RT activity in CHO cells as expected from reports in the literature (for example, Anderson, K. P. et al., Virology 181(1): 305-311 (1991)). With these cells as positive control and human 293 cells free of retroviral activity as negative control a bracket is defined that allows interpretation of unknown RT activity in the supernatant of cell cultures (figure 4, bold squares and bold triangles).

We found moderate RT-activity in chicken embryo fibroblasts (figure 4, bold diamond symbols).

The signal for RT activity in the duck cell supernatant was congruent with the signal for RT activity in 293 cells, and both again congruent with a control representing the detection limit for our assay consisting of model RNA not incubated with RT (figure 4, compare curves with open and bold triangles and

grey circles). Equivalent levels of signal intensity (delta Rn) were separated by at least two cycle numbers between samples from CHO cells and chicken embryo fibroblasts (that for these experiments are derived from a source known to be only weakly RT-positive) and by at least four cycle numbers between samples from CHO cells and the 293 negative control and the duck cell culture. Thus, contrary to chicken cells the described duck cells do not exhibit RT activity and thus fulfill an essential attribute for suitability in pharmaceutical applications.

Example 5: Modified vaccinia virus Ankara (MVA)

Suitability of the expanded foci as substrate for amplification of MVA was determined for liver, retina, somites and extra-embryonic membrane lines. Table 1 and figure 5 show results obtained by infection of the cell lines with an inoculum prepared from a large scale preparation of MVA (ATCC #VR-1508) on CEFp, primary chicken embryonic fibroblasts. The data in the table obtained by infection with an MOI (multiplicity of infection or number of infectious particles per host cell) of 0.1 demonstrates that viral output of retina and somite cells (in plaque forming units per ml) are comparable to or even exceed the output obtained with CEFp cells.

	MVA yield in pfu/ml (after 48 h, infection with MOI of 0.1)
CEFp	3.54×10^7
retina	2.06×10^7
liver	3.20×10^4
somite	4.60×10^7
membrane	4.03×10^3

Table 1: Comparison of virus titers obtained in parallel infections of 1 to 5×10^5 cells in cavities of 24-well plates. Input virus was adjusted for an MOI of 0.1. CEFp, fresh primary chicken embryonic fibroblasts; membrane, extra-embryonic membrane.

Plaque-forming units for MVA on duck cells were determined as follows: MVA virus was recovered from infected cells after 48 hours from the supernatant and from adherent cells opened by repeated freeze-thawing. VERO (African green monkey kidney) cells were seeded in 96 well plates (2×10^4 cells per well) and

infected with serial 10-fold dilutions of MVA-containing suspension on the following day. Two days thereafter, the cultures were fixed with methanol and infected cells incubated with polyclonal vaccinia virus antibodies (Quartett, Germany, #9503-2057, at 1:1000 dilution in PBS containing 1% fetal calf serum) for 1 hour at 37°C. Two wash steps were performed with PBS containing 0.05% Tween 20 (Sigma Corp, USA) and secondary antibody to the vaccinia-specific antibody is added at 1:1000 dilution in PBS containing 1% fetal calf serum. This secondary antibody is coupled to the peroxidase enzyme that catalyzes a color reaction upon incubation with AEC reagent (3-amino-9-ethyl-carbozole; 0.3 mg/ml in 0.1 M acetate buffer pH 5.0 containing 0.015% H_2O_2). Infected foci are identified by light microscopy and plaque forming units are calculated from the maximum dilution of MVA suspension that yields a positive dye reaction.

Figure 5 depicts the output of virus per cell. The output of virus per cell correlates with permissiveness of a given host cell for a particular virus. Permissiveness is influenced by biochemical properties such as receptor density or efficiency of processing of viral structural proteins. Figure 5 demonstrates that the number of infectious particles released per retina cell or per somite cell compares favourably with the obtained infectious particles per chicken embryonic fibroblast.

Division of "output virus per cell" by the "MOI" yields the burst size, the ratio of input virus to output virus. Burst size is equivialent to amplification of virus and thus important to estimate cost and required resources for large scale production. The determined burst sizes in the described example are 374 for CEFp, 513 for retina cells, and 1108 for somite-derived cells. Retina cells and somite cells yield better values than fresh primary chicken embryo fibroblasts and thus should provide superior substrates for large scale production of MVA.

The unsatisfactory results for MVA amplification obtained with cells derived from liver or extra-embryonic membrane cannot be extended to other virus families: it is evident to one familiar with the art that amplification of other viruses, for example vaccine-relevant influenza viruses; may be extremely successful on these cells.

It is conceivable that with subsequent passaging of virus on a given host cell the output titer decreases. Such events may occur if host cells support most but not all steps in the various stages of the infectious cycle. To address this question serial passage of MVA was performed on duck retina cells transformed with plasmid 49E. The data in figure 6 demonstrate that MVA is not lost with passaging on these cells: at similar levels of input virus adjusted to an MOI of 0.3 (given by bars in figure 6) the burst size (bold squares) increases nine-fold from 35 to 315. The reason for the increase in burst size may be due to to improved properties of the host cell as passage number increases.

In conclusion, duck retina and somite-derived cells obtained by transfection of Ad5-E1 region under cGMP conditions stably support amplification of MVA with an efficiency comparable to or better than primary chicken embryonic fibroblasts. Due to the highly attenuated nature of MVA conventional cell lines for large-scale production of viruses are not suitable. It is a surprising finding that designed duck cell lines performed better than primary chicken cells in propagation of MVA and thus are able to provide novel production platforms for this important vaccine candidate. The described cell lines were generated under cGMP conditions and are therefore suitable for pharmaceutical application.